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RAFFINOSE SYNTHASE GENES AND THEIR USE

FIELD OF INVENTION

The present invention relates to raffinose synthase genes and their use.

BACKGROUND OF THE INVENTION

Raffinose family oligosaccharides are derivatives of sucrose, which are represented by o-α-D-galactopyranosyl-(1$\rightarrow$6) n-o-α-D-glucopyranosyl-(1$\rightarrow$2)-β-D-flucto-furanoside as the general formula, and they are designated "raffinose" when $n = 1$, "stachyose" when $n = 2$, "verbascose" when $n = 3$, and "ajugose" when $n = 4$.

The greatest contents of such raffinose family oligosaccharides are found in plants, except for sucrose, and it has been known that they are contained not only in higher plants including gymnosperms such as pinaceous plants (e.g., spruce) and angiosperms such as leguminous plants (e.g., soybean, kidney bean), brassicaceous plants (e.g., rape), chenopodiaceous plants (e.g., sugar beet), malvaceous plants (e.g., cotton), and salicaceous plants (e.g., poplar), but also in green algae, chlorella. Thus, they occur widely in the plant kingdom similarly to sucrose.

Raffinose family oligosaccharides play a role as reserve sugars in the storage organs or seeds of many plants or as translocating sugars in the phenomenon of sugar transportation between the tissues of some plants.

Furthermore, it has been known that raffinose family oligosaccharides have an effect of giving good conditions of enterobacterial flora, if present at a suitable amount in food. Therefore, raffinose family oligosaccharides have already been used as a functional food material for addition to some kinds of food and utilized in the field of specified healthful food.

Raffinose family oligosaccharides having such a role and utility are produced by the raffinose oligosaccharide synthesis system beginning with sucrose in many plants. This biosynthesis system usually involves a reaction for the sequential addition of galactosyl groups from galactotinol through an α(1$\rightarrow$6) bond to a hydroxyl group

attached to the carbon atom at position 6 of a D-glucose residue in a sucrose molecule.

In the first step of this biosynthesis system, raffinose synthase is an enzyme concerned in the reaction of raffinose production by combining a D-galactosyl group from galactotinol through an $\alpha(1\rightarrow6)$ bond with a hydroxyl group attached to the carbon atom at position 6 of a D-glucose residue in a sucrose molecule. It has been suggested that this enzyme constitutes a rate limiting step in the above synthesis system, and it has been revealed that this enzyme is quite important in the control of biosynthesis of raffinose family oligosaccharides.

The control of expression level or activity of raffinose synthase in plants makes it possible to change the contents of raffinose family oligosaccharides in these plants. However, raffinose synthase, although the presence of this enzyme itself was already confirmed in many plants by the measurement of its activity with a biochemical technique, has not yet been successfully isolated and purified as a homogeneous protein. In addition, the amino acid sequence of this enzyme is still unknown, and no report has been made on an attempt at beginning to isolate a gene coding for this enzyme.

SUMMARY OF THE INVENTION

Under these circumstances, the present inventors have intensively studied and finally succeeded in isolating a raffinose synthase and a gene coding for this enzyme from broad bean, thereby completing the present invention.

Thus, the present invention provides the following:

1) A raffinose synthase gene isolated from a plant and having a nucleotide sequence coding for an amino acid sequence of a protein capable of producing raffinose by combining a D-galactosyl group through an $\alpha(1\rightarrow6)$ bond with a hydroxyl group attached to the carbon atom at position 6 of a D-glucose residue in a sucrose molecule.

2) The raffinose synthase gene according to item 1, wherein the plant is a dicotyledon.

3) The raffinose synthase gene according to item 2, wherein the dicotyle-

don is a leguminous plant.

4) The raffinose synthase gene according to item 3, wherein the leguminous plant is broad bean.

5) A raffinose synthase gene having a nucleotide sequence coding for protein (a) or (b) as defined below:

(a) protein having the amino acid sequence of SEQ ID NO:1;

(b) protein having an amino acid sequence derived by deletion, replacement, modification or addition of one or several amino acids in the amino acid sequence of SEQ ID NO:1, and capable of producing raffinose by combining a D-galactosyl group through an $\alpha(1\rightarrow6)$ bond with a hydroxyl group attached to the carbon atom at position 6 of a D-glucose residue in a sucrose molecule.

6) A raffinose synthase gene having the nucleotide sequence of SEQ ID NO:2.

7) The raffinose synthase gene according to item 3, wherein the leguminous plant is soybean.

8) A raffinose synthase gene having a nucleotide sequence coding for protein (a) or (b) as defined below:

(a) protein having the amino acid sequence of SEQ ID NO:3;

(b) protein having an amino acid sequence derived by deletion, replacement, modification or addition of one or several amino acids in the amino acid sequence of SEQ ID NO:3, and capable of producing raffinose by combining a D-galactosyl group through an $\alpha(1\rightarrow6)$ bond with a hydroxyl group attached to the carbon atom at position 6 of a D-glucose residue in a sucrose molecule.

9) A raffinose synthase gene having the nucleotide sequence of SEQ ID NO:4.

10) The raffinose synthase gene according to item 2, wherein the dicotyledon is a lamiaceous plant.

11) The raffinose synthase gene according to item 10, wherein the lamia-

ceous plant is Japanese artichoke.

12) A raffinose synthase gene having a nucleotide sequence coding for the amino acid sequence of SEQ ID NO:5.

13) A raffinose synthase gene having the nucleotide sequence of SEQ ID NO:6.

14) The raffinose synthase gene according to item 1, wherein the plant is a monocotyledon.

15) The raffinose synthase gene according to item 14, wherein the monocotyledon is a gramineous plant.

16) The raffinose synthase gene according to item 15, wherein the gramineous plant is corn.

17) A raffinose synthase gene having a nucleotide sequence coding for the amino acid sequence of SEQ ID NO:7.

18) A raffinose synthase gene having the nucleotide sequence of SEQ ID NO:8.

19) A raffinose synthase protein having amino acid sequence (a) or (b) as defined below:

(a) amino acid sequence of SEQ ID NO:1 or SEQ ID NO:3;

(b) amino acid sequence derived by deletion, replacement, modification or addition of one or several amino acids in the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:3;

the protein being capable of producing raffinose by combining a D-galactosyl group through an $\alpha(1\rightarrow6)$ bond with a hydroxyl group attached to the carbon atom at position 6 of a D-glucose residue in a sucrose molecule.

20) A raffinose synthase protein having the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:3.

21) A gene fragment having a partial nucleotide sequence of the raffinose synthase gene of item 1, 2, 3, 4, 7, 10, 11, 14, 15 or 16.

22) A gene fragment having a partial nucleotide sequence of the raffinose synthase gene of item 5, 6, 8, 9, 12, 13, 17 or 18.

23) The gene fragment according to item 21 or 22, wherein the number of nucleotides is in the range of from 15 to 50.

24) A method for the detection of a raffinose synthase gene or a gene fragment having a partial nucleotide sequence thereof, which comprises hybridizing a probe of the labeled gene fragment of item 21, 22 or 23 to an organism-derived genomic DNA or cDNA fragment; and detecting the DNA fragment bound specifically to the probe.

25) A method for the detection of a raffinose synthase gene or a gene fragment having a partial nucleotide sequence thereof, which comprises hybridizing a probe of the labeled gene fragment of item 21, 22 or 23 to a plant-derived genomic DNA or cDNA fragment; and detecting the DNA fragment bound specifically to the probe.

26) A method for the amplification of a raffinose synthase gene or a gene fragment having a partial nucleotide sequence thereof, which comprises annealing a primer having a nucleotide sequence of the gene fragment of item 21, 22 or 23 to organism-derived genomic DNA or cDNA; and amplifying the resulting DNA fragment by polymerase chain reaction.

27) A method for the amplification of a raffinose synthase gene or a gene fragment having a partial nucleotide sequence thereof, which comprises annealing a primer having a nucleotide sequence of the gene fragment of item 21, 22 or 23 to plant-derived genomic DNA or cDNA; and amplifying the resulting DNA fragment by polymerase chain reaction.

28) A method for obtaining a raffinose synthase gene, comprising the steps of identifying a DNA fragment containing a raffinose synthase gene or a gene fragment having a partial nucleotide sequence thereof by the method of item 24, 25, 26 or 27; and isolating and purifying the DNA fragment identified.

29) A raffinose synthase gene obtained by identifying a DNA fragment

containing a raffinose synthase gene or a gene fragment having a partial nucleotide sequence thereof by the method of item 24, 25, 26 or 27; and isolating and purifying the DNA fragment identified.

30) A chimera gene comprising the raffinose synthase gene of item 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18 or 29, and a promoter linked thereto.

31) A transformant obtained by introducing the chimera gene of item 30 into a host organism.

32) A plasmid comprising the raffinose synthase gene of item 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 29 or 30.

33) A host organism transformed with the plasmid of item 32, or a cell thereof.

34) A microorganism transformed with the plasmid of item 32.

35) A plant transformed with the plasmid of item 32, or a cell thereof.

36) A method for metabolic modification, which comprises introducing the raffinose synthase gene of item 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 29 or 30 into a host organism or a cell thereof, so that the content of raffinose family oligosaccharides in the host organism or the cell thereof is changed.

37) A method for the production of a raffinose synthase protein, which comprises isolating and purifying a raffinose synthase protein from a culture obtained by cultivating the microorganism of item 34.

38) An anti-raffinose synthase antibody capable of binding to the raffinose synthase protein of item 19 or 20.

39) A method for the detection of a raffinose synthase protein, which comprises treating a test protein with the anti-raffinose synthase antibody of item 38; and detecting the raffinose synthase protein by antigen-antibody reaction between the antibody and the raffinose synthase protein.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the construction of plasmids used for the expression of a raffinose synthase gene in *Escherichia coli*. pBluescriptKS-RS is a plasmid containing the raffinose synthase gene cloned therein. RS represents the raffinose synthase gene, and the nucleotide sequences shown in the upper portion of this figure are those of both terminal portions of the raffinose synthase gene. A partial sequence represented by small letters is a nucleotide sequence derived from the vector pBluescriptII KS-. Two boxed nucleotide sequences are the initiation codon (ATG) and termination codon (TGATAA) of the raffinose synthase gene, respectively. The recognition sites for several restriction endonucleases are shown above the nucleotide sequences. pGEX-RS and pTrc-RS are plasmids used for the expression of the raffinose synthase gene in *E. coli*. Ptac, Ptrc, GST, $lacI^q$, and rrnB represent tac promoter, trc promoter, glutathione-S-transferase gene, lactose repressor gene, and termination signal for the transcription of ribosomal RNA, respectively.

Figure 2 shows the construction of expression vectors used for the expression in plants of chimera genes each having a raffinose synthase gene and a promoter linked thereto. The restriction endonuclease map of the raffinose synthase gene cloned in the plasmid pBluescriptKS-RS is shown in the lower portion of this figure. pBI221RS and pBI221(–)RS indicate the restriction endonuclease maps of expression vectors used for the transformation of soybean. 35S and NOS represent 35S promoter derived from cauliflower mosaic virus and nopaline synthase gene terminator, respectively.

Figure 3 shows the construction of expression vectors used for the expression in plants of chimera genes each having a raffinose synthase gene and a promoter linked thereto. The restriction endonuclease map of the raffinose synthase gene cloned in the plasmid pBluescriptKS-RS is shown in the upper portion of this figure. pBI121RS and pBI121(–)RS indicate the restriction endonuclease maps of binary vectors used for the transformation of mustard. For the binary vector, only a region between the right border and the left border is shown. 35S, NOS and NPT represent 35S promoter derived from

cauliflower mosaic virus, nopaline synthase gene terminator and kanamycin resistance gene, respectively.

DETAILED DESCRIPTION OF THE INVENTION

The gene engineering methods described below can be carried out according to ordinary methods, for example, as described in "Molecular Cloning: A Laboratory Manual 2nd edition" (1989), Cold Spring Harbor Laboratory Press, ISBN 0-87969-309-6; "Current Protocols In Molecular Biology" (1987), John Wiley & Sons, Inc. ISBN 0-471-50338-X; and "Current Protocols In Protein Science" (1995), John Wiley & Sons, Inc. ISBN 0-471-11184-8.

The term "raffinose synthase gene" as used herein refers to a gene having a nucleotide sequence coding for the amino acid sequence of a protein capable of producing raffinose by combining a D-galactosyl group through an $\alpha(1\rightarrow6)$ bond with a hydroxyl group attached to the carbon atom at position 6 of a D-glucose residue in a sucrose molecule (hereinafter referred to simply as the present gene), and such a gene can be prepared, for example, from plants.

More specifically, the present gene can be prepared from dicotyledons such as leguminous plants (e.g., broad bean, soybean) and lamiaceous plants (e.g., Japanese artichoke) or from monocotyledons such as gramineous plants (e.g., corn). Specific examples of the present gene are a "raffinose synthase gene having a nucleotide sequence coding for a protein having the amino acid sequence of SEQ ID NO:1"; a "raffinose synthase gene having a nucleotide sequence coding for a protein having an amino acid sequence derived by deletion, replacement, modification or addition of one or several amino acids in the amino acid sequence of SEQ ID NO:1, and capable of producing raffinose by combining a D-galactosyl group through an $\alpha(1\rightarrow6)$ bond with a hydroxyl group attached to the carbon atom at position 6 of a D-glucose residue in a sucrose molecule"; a "raffinose synthase gene having a nucleotide sequence coding for a protein having the amino acid sequence of SEQ ID NO:3"; a "raffinose synthase gene having a

nucleotide sequence coding for a protein having an amino acid sequence derived by deletion, replacement, modification or addition of one or several amino acids in the amino acid sequence of SEQ ID NO:3, and capable of producing raffinose by combining a D-galactosyl group through an $\alpha(1\rightarrow6)$ bond with a hydroxyl group attached to the carbon atom at position 6 of a D-glucose residue in a sucrose molecule"; a "raffinose synthase gene having a nucleotide sequence coding for the amino acid sequence of SEQ ID NO:5"; and a "raffinose synthase gene having a nucleotide sequence coding for the amino acid sequence of SEQ ID NO:7."

The present gene can be obtained, for example, by the following method.

The tissues of a leguminous plant such as broad bean (*Vicia faba*) or soybean (*Glycine max*) are frozen in liquid nitrogen and ground physically with a mortar or other means into finely powdered tissue debris. From the tissue debris, RNA is extracted by an ordinary method. Commercially available RNA extraction kits can be utilized in the extraction. The whole RNA is separated from the RNA extract by ethanol precipitation. From the whole RNA separated, poly-A tailed RNA is fractionated by an ordinary method. Commercially available oligo-dT columns can be utilized in the fractionation. cDNA is synthesized from the fraction obtained (i.e., poly-A tailed RNA) by an ordinary method. Commercially available cDNA synthesis kits can be utilized in the synthesis.

For example, cDNA fragments of the "raffinose synthase gene having a nucleotide sequence coding for a protein having the amino acid sequence of SEQ ID NO:1" as the present gene can be obtained by PCR amplification using the broad bean-derived cDNA obtained above as a template and primers 1 to 3 shown in list 1 below. The primers used therein can be designed and synthesized on the basis of the nucleotide sequence of SEQ ID NO:2, depending upon the purpose. For example, in order to amplify the open reading frame region of the "raffinose synthase gene having a nucleotide sequence coding for a protein having the amino acid sequence of SEQ ID NO:1," primers 1 to 4 shown in list 2 below may be designed and synthesized.

In the same manner, cDNA fragments of the "raffinose synthase gene having

a nucleotide sequence coding for a protein having the amino acid sequence of SEQ ID NO:3" can be obtained by PCR amplification with the soybean-derived cDNA obtained above as a template and, for example, primers 4 to 6 shown in list 1 below. The primers used therein can be designed and synthesized on the basis of the nucleotide sequence of SEQ ID NO:4, depending upon the purpose. For example, in order to amplify the open reading frame region of the "raffinose synthase gene having a nucleotide sequence coding for a protein having the amino acid sequence of SEQ ID NO:3," primers 5 to 8 shown in list 2 below may be designed and synthesized.

The amplified DNA fragments can be subcloned according to ordinary methods, for example, as described in "Molecular Cloning: A Laboratory Manual 2nd edition" (1989), Cold Spring Harbor Laboratory Press; and "Current Protocols In Molecular Biology" (1987), John Wiley & Sons, Inc. ISBN 0-471-50338-X. More specifically, cloning can be effected, for example, using a TA cloning kit (Invitrogen) and a plasmid vector such as pBluescript II (Stratagene). The nucleotide sequences of the DNA fragments cloned can be determined by the dideoxy terminating method, for example, as described by F. Sanger, S. Nicklen, A.R. Coulson, Proceedings of National Academy of Science U.S.A. (1977), 74, pp. 5463-5467. For example, ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit commercially available from Perkin-Elmer may preferably be used.

(List 1)

Primer 1: AATTTTCAAG CATAGCCAAG TTAACCACCT 30 mer (SEQ ID NO:9)
Primer 2: GCTCACAAGA TAATGATGTT AGTC 24 mer (SEQ ID NO:10)
Primer 3: ATACAAGTGA GGAACTTGAC CA 22 mer (SEQ ID NO:11)
Primer 4: CCAAACCATA GCAAACCTAA GCAC 24 mer (SEQ ID NO:12)
Primer 5: ACAACAGAAA AATATGACTC TTATTACT 28 mer (SEQ ID NO:13)
Primer 6: AAAAGAGAGT CAAACATCAT AGTATC 26 mer (SEQ ID NO:14)

(List 2)

Primer 1: ATGGCACCAC CAAGCATAAC CAAAACTGC 29 mer (SEQ ID NO:15)
Primer 2: ATGGCACCAC CAAGCATAAC CAAAACTGCA ACCCTCCAAG ACG 43 mer (SEQ ID NO:16)

Primer 3: TCAAAATAAA AACTGGACCA AAGAC 25 mer (SEQ ID NO:17)

Primer 4: TCAAAATAAA AACTGGACCA AAGACAATGT 30 mer (SEQ ID NO:18)

Primer 5: ATGGCTCCAA GCATAAGCAA AACTG 25 mer (SEQ ID NO:19)

Primer 6: ATGGCTCCAA GCATAAGCAA AACTGTGGAA CT 32 mer (SEQ ID NO:20)

Primer 7: TCAAAATAAA AACTCAACCA TTGAC 25 mer (SEQ ID NO:21)

Primer 8: TCAAAATAAA AACTCAACCA TTGACAATTT TGAAGCACT 39 mer (SEQ ID NO.22)

The term "gene fragment" as used herein refers to a gene fragment having a partial nucleotide sequence of the present gene (hereinafter referred to simply as the present gene fragment). For example, it may be a gene fragment derived from a plant and having a partial nucleotide sequence of the gene having a nucleotide sequence coding for a protein capable of producing raffinose by combining a D-galactosyl group through an $\alpha(1\rightarrow6)$ bond with a hydroxyl group attached to the carbon atom at position 6 of a D-glucose residue in a sucrose molecule. Specific examples of the present gene fragment are a gene fragment having a partial nucleotide sequence of the gene having a nucleotide sequence coding for the amino acid sequence of SEQ ID NO:1 and a gene fragment having a partial nucleotide sequence of the gene having a nucleotide sequence of SEQ ID NO:2, more specifically a gene fragment having a nucleotide sequence or a partial nucleotide sequence thereof, coding for any of the amino acid sequences shown in list 3 below.

These gene fragments can be used as probes in the hybridization method or as primers in the PCR method. For the primers in the PCR method, it is generally preferred that the number of nucleotides is greater from a viewpoint that the specificity of annealing is ensured; it is, however, also preferred that the number of nucleotides is not so great from viewpoints that the primers themselves are liable to have a higher structure giving possible deterioration of the annealing efficiency and that complicated procedures are required in the purification after the synthesis. In usual cases, preferred is a gene fragment consisting of single-stranded DNA, wherein the number of nucleotides is in the range of from 15 to 50.

(List 3)

#1 Gly Ile Lys Phe Met Ser Ile Phe Arg Phe Lys Val Trp Trp Thr Thr His Trp Val Gly (SEQ ID NO:23)

#2 Ile Ile Asp Lys Phe Gly Trp Cys Thr Trp Asp Ala Phe Tyr (SEQ ID NO:24)

#3 Gly Gly Cys Pro Pro Gly Phe Val Ile Ile Asp Asp Gly Trp Gln (SEQ ID NO:25)

#4 Thr Ser Ala Gly Glu Gln Met Pro Cys Arg Leu Val Lys Tyr Glu Glu Asn (SEQ ID NO:26)

#5 Val Tyr Val Trp His Ala Leu Cys Gly Tyr Trp Gly Gly Val Arg Pro (SEQ ID NO:27)

#6 Thr Met Glu Asp Leu Ala Val Asp Lys Ile Val Glu Asn Gly Val Gly Leu Val Pro Pro (SEQ ID NO:28)

#7 Gly Leu His Ser His Leu Glu Ser Ala Gly Ile Asp Gly Val Lys Val Asp Val Ile His Leu Leu Glu (SEQ ID NO:29)

#8 Gly Gly Arg Val Glu Leu Ala Arg Ala Tyr Tyr Lys Ala Leu (SEQ ID NO:30)

#9 Val Lys Lys His Phe Lys Gly Asn Gly Val Ile Ala (SEQ ID NO:31)

#10 Glu His Cys Asn Asp Phe Phe Leu Leu Gly Thr Glu Ala Ile Ser Leu Gly Arg Val Gly Asp Asp Phe Trp Cys Ser Asp Pro Ser Gly Asp Pro Asn Gly Thr Tyr Trp Leu Gln Gly Cys His Met Val His Cys (SEQ ID NO:32)

#11 Ala Tyr Asn Ser Leu Trp Met Gly Asn Phe Ile Gln Pro Asp Trp Asp Met Phe Gln Ser Thr His Pro Cys Ala Glu Phe His Ala Ala Ser Arg Ala Ile Ser Gly Gly Pro Ile Tyr Val Ser Asp (SEQ ID NO: 33)

#12 Leu Pro Asp Gly Ser Ile Leu Arg Cys (SEQ ID NO: 34)

#13 Ala Leu Pro Thr Arg Asp Cys Leu Phe Glu Asp Pro Leu His Asn Gly Lys Thr Met Leu Lys Ile Trp Asn (SEQ ID NO: 35)

#14 Gly Val Leu Gly Leu Phe Asn Cys Gln Gly Gly Gly Trp (SEQ ID NO: 36)

#15 Phe Ala Pro Ile Gly Leu Val Asn Met (SEQ ID NO: 37)

The present gene fragment is labeled, and then used as a probe in the hybridization method and hybridized to organism-derived DNA, so that a DNA fragment having the probe specifically bound thereto can be detected. Thus, from an organism-derived gene library, a raffinose synthase gene having a nucleotide sequence coding for the amino acid sequence of an enzyme capable of producing raffinose by combining a D-galactosyl group through an $\alpha(1\rightarrow6)$ bond with a hydroxyl group attached to the carbon atom at position 6 of a D-glucose residue in a sucrose molecule; or a gene fragment having a partial nucleotide sequence thereof, can be detected (hereinafter referred to simply as the present detection method).

As the organism-derived DNA, for example, a cDNA library or a genomic DNA library of a desired plant can be used. The gene library may also be a commercially available gene library as such or a library prepared according to an ordinary library preparation method, for example, as described in "Molecular Cloning: A Laboratory Manual 2nd edition" (1989), Cold Spring Harbor Laboratory Press; "Current Protocols In Molecular Biology" (1987), John Wiley & Sons, Inc. ISBN 0-471-50338-X.

As the hybridization method, plaque hybridization or colony hybridization can be employed, depending upon the kind of vector used in the preparation of a library. More specifically, when a library to be used is constructed with a phage vector, a suitable host microorganism is mixed with the phage under infectible conditions, which is further mixed with a soft agar medium, and the mixture is plated on an agar medium. Thereafter, a culture is grown at 37°C until a plaque of an appropriate size appears. When a library to be used is constructed with a plasmid vector, the plasmid is transformed in a suitable host microorganism to form a transformant. The transformant obtained is diluted to a suitable concentration, and the dilution is plated on an agar medium, after which a culture is grown at 37°C until a colony of an appropriate size appears.

In either case of the above libraries, a membrane filter is placed on the surface of the agar medium after the cultivation, so that the phage or transformant is transferred to the membrane. This membrane is denatured with an alkali, followed by neutralization, and for example, when a nylon membrane is used, the membrane is irradiated with ultraviolet light, so that DNA is fixed on the membrane. This membrane is then subjected to hybridization with the present gene fragment labeled by an ordinary method as a probe. For this method, reference may be made, for example, to D.M. Glover ed., "DNA cloning, a practical approach" IRL PRESS (1985), ISBN 0-947946-18-7. There are various reagents and temperature conditions to be used in the hybridization; for example, prehybridization is carried out by the addition of 6 x SSC (0.9 M NaCl, 0.09 M citric acid), 0.1-1% SDS, 100 μg/ml denatured salmon sperm DNA, and incubation at 65°C for 1 hour. The present gene fragment labeled is then added as a probe, and mixed.

Hybridization is carried out at 42-68°C for 4 to 16 hours. The membrane is washed with 2 x SSC, 0.1-1% SDS, further rinsed with 0.2 x SSC, 0-0.1% SDS, and then dried. The membrane is analyzed, for example, by autoradiography or other techniques, to detect the position of the probe on the membrane and thereby detect the position of DNA having a nucleotide sequence homologous to that of the probe used. Thus, the present gene or the present gene fragment can be detected. The clone corresponding to the position of DNA thus detected on the membrane is identified on the original agar medium, and the positive clone is selected, so that the clone having the DNA can be isolated. The same procedures of detection are repeated to purify the clone having the DNA.

Other detection methods can also be used, for example, GENE TRAPPER cDNA Positive Selection System Kit commercially available from Gibco BRL. In this method, a single-stranded DNA library is hybridized with the present gene fragment biotinylated (i.e., probe), followed by the addition of streptoavidin-bound magnet beads and mixing. From the mixture, the streptoavidin-bound magnetic beads are collected with a magnet, so that single-stranded DNA having a nucleotide sequence homologous to that of the probe used, which has been bound to these beads through the present gene fragment, biotin and streptoavidin, is collected and detected. Thus, the present gene or the present gene fragment can be detected. The single-stranded DNA collected can be changed into a double-strand form by treatment with a suitable DNA polymerase using a suitable oligonucleotide as a primer.

The present detection method may also be used in the analysis of a plant. More specifically, plant genomic DNA is prepared according to an ordinary method, for example, as described in "Cloning and Sequence (Plant Biotechnology Experiment Manual)" complied under the supervision of Itaru Watanabe, edited by Masahiro Sugiura, published by Noson Bunka-sha, Tokyo (1989). The plant genomic DNA is digested with several kinds of suitable restriction endonucleases, followed by electrophoresis, and the electrophoresed DNA is blotted on a filter according to an ordinary method. This filter is subjected to hybridization with a probe prepared from the present gene fragment by an

ordinary method, and DNA fragments to which the probe hybridizes are detected. The DNA fragments detected are compared in length between different varieties of a specified plant species. The differences in length make possible the analysis of differences in phenotypic characteristics accompanied with the expression of raffinose family oligo-saccharides between these varieties. Furthermore, when the DNA fragments detected by the above method are compared in length between the gene recombinant plant and the non-gene recombinant plant of the same variety, the former plant can be discriminated from the latter plant by the detection of hybridizing bands greater in number or higher in concentration for the former plant than for the latter plant. This method can be carried out according to the RFLP (restriction fragment length polymorphism) method, for example, as described in "Plant PCR Experiment Protocols" complied under the supervision of Ko Shimamoto and Takuji Sasaki, published by Shujun-sha, Tokyo (1995), ISBN 4-87962-144-7, pp. 90-94.

The PCR method using a primer having the nucleotide sequence of the present gene fragment makes it possible to amplify from organism-derived DNA, a raffinose synthase gene having a nucleotide sequence coding for the amino acid sequence of an enzyme capable of producing raffinose by combining a D-galactosyl group through an $\alpha(1\rightarrow6)$ bond with a hydroxyl group attached to the carbon atom at position 6 of a D-glucose residue in a sucrose molecule; or a gene fragment having a partial nucleotide sequence thereof (hereinafter referred to simply as the present amplification method).

More specifically, for example, an oligonucleotide having 15 to 50 nucleotides in the nucleotide sequence of the present gene fragment at the 3'-terminus is chemically synthesized by an ordinary synthesis method. Based on the codon table below, showing the correspondence of amino acids encoded in nucleotide sequences, a mixed primer can also be synthesized so that a residue at a specified position in the primer is changed to a mixture of several bases, depending upon the variation of codons which can encode a certain amino acid.

CODON TABLE

Phe	UUU	Ser	UCU	Tyr	UAU	Cys	UGU
	UUC		UCC		UAC		UGC
Leu	UUA		UCA	Stop	UAA	Stop	UGA
	UUG		UCG		UAG	Trp	UGG
	CUU	Pro	CCU	His	CAU	Arg	CGU
	CUC		CCC		CAC		CGC
	CUA		CCA	Gln	CAA		CGA
	CUG		CCG		CAG		CGG
Ile	AUU	Thr	ACU	Asn	AAU	Ser	AGU
	AUC		ACC		AAC		AGC
	AUA		ACA	Lys	AAA	Arg	AGA
Met	AUG		ACG		AAG		AGG
Val	GUU	Ala	GCU	Asp	GAU	Gly	GGU
	GUC		GCC		GAC		GGC
	GUA		GCA	Glu	GAA		GGA
	GUG		GCG		GAG		GGG

Furthermore, a base capable of forming a pair with plural kinds of bases, such as inosine, can also be used instead of the above mixture of several bases. More specifically, for example, primers having nucleotide sequences as shown in list 4 can be used. In this context, an oligonucleotide having the same nucleotide sequence as the coding strand of the present gene consisting of double-stranded DNA is designated "sense primer," and an oligonucleotide having a nucleotide sequence complementary to the coding strand, "antisense primer."

A sense primer having the same nucleotide sequence as present on the 5'-upstream side in the coding strand of a raffinose synthase gene or a gene fragment having a partial nucleotide sequence thereof to be amplified, and an antisense primer having a nucleotide sequence complementary to the nucleotide sequence on the 3'-downstream side in this coding strand, are used in combination for PCR reaction to amplify a DNA fragment, for example, with a gene library, genomic DNA or cDNA as a template. At this time, the amplification of a DNA fragment can be confirmed by an

ordinary method with electrophoresis. For the DNA fragment amplified, its restriction endonuclease map is constructed or its nucleotide sequence is determined by an ordinary method, so that the present gene or the present gene fragment can be identified. As the gene library used herein, for example, a cDNA library or a genomic cDNA library of a desired plant can be used. For the plant gene library, a commercially available library derived from plant can be used as such; or a library prepared according to an ordinary library preparation method, for example, as described in "Molecular Cloning: A Laboratory Manual 2nd edition" (1989), Cold Spring Harbor laboratory Press or "Current Protocols in Molecular Biology" (1987), John Wiley & Sons, Inc., ISBN 0-471-50338-X, can also be used. As the genomic DNA or cDNA used in the present amplification method, for example, cDNA or genomic cDNA prepared from a desired plant can be used.

More specifically, for example, a primer designed on the amino acid sequence of SEQ ID NO:1 is used for the present amplification method with cDNA derived from Japanese artichoke, which is a lamiaceous plant, as a template, so that a raffinose synthase gene fragment having the nucleotide sequence of SEQ ID NO:6 can be amplified. Furthermore, for example, a primer designed on the amino acid sequence of SEQ ID NO:1 is used for the present amplification method with cDNA derived from corn, which is a gramineous plant, as a template, so that a raffinose synthase gene fragment having the nucleotide sequence of SEQ ID NO:8 can be amplified.

(List 4)

1-F 32mer (SEQ ID NO: 38)

rz TTIAAIGTITGGTGGACIACICAITGGGTIGG

2-F 41mer (SEQ ID NO: 39)

rz ATIATIGAIAAITTIGGITGGTGIACITGGGAIGCITTITA

2-RV 41mer (SEQ ID NO: 40)

rz TAIAAIGCITCCCAIGTICACCAICCIAAITTITCIATIAT

3-F 44mer (SEQ ID NO: 41)

rz GGIGGITGICCICCIGGITTIGTIATIATIGAIGAIGGITGGCA

3-RV	44mer TGCCAICCITCITCIATIATIACIAAICCIGGIGGICAICCICC	(SEQ ID NO: 42)
4-F	32mer AAIAAICAITTIAAIGGIAAIGGIGTIATIGC	(SEQ ID NO: 43)
4-RV	32mer GCIATIACICCITTICCITTIAAITGITTITT	(SEQ ID NO: 44)
5-F	38mer TGGATGGGIAAITTIATICAICCIGAITGGGAIATGTT	(SEQ ID NO: 45)
5-RV	38mer AACATITCCCAITCIGGITGIATIAAITTICCCATCCA	(SEQ ID NO: 46)
6-RV	27mer CATITTIACIA(AG)ICCIATIGGIGCIAA	(SEQ ID NO: 47)

The present amplification method can also be utilized for the analysis of a plant gene. More specifically, for example, plant genomic DNA prepared from different varieties of a specified plant species is used as a template for the present amplification method to amplify a DNA fragment. The DNA fragment amplified is mixed with a solution of formaldehyde, which is denatured by heating at 85°C for 5 minutes, followed by rapid cooling on ice. This sample is subjected to electrophoresis, for example, on a 6% polyacrylamide gel containing 0% or 10% glycerol. In this electrophoresis, a commercially available electrophoresis apparatus for SSCP (single strand conformation polymorphism) can be used, and electrophoresis is carried out, while the gel is kept at a constant temperature, e.g., 5°C, 25°C or 37°C. From the electrophoresed gel, a DNA fragment is detected, for example, by a method such as silver staining method with commercially available reagents.

From the differences of behavior between the varieties in the electrophoresis of the DNA fragment detected, a mutation in the raffinose synthase gene is detected, and an analysis is carried out for differences caused by the mutation in phenotypic characteristics accompanied with the expression of raffinose family oligosaccharides. This method can be carried out according to the SSCP method, for example, as described in "Plant PCR Experiment Protocols" complied under the supervision of Ko Shimamoto and Takuji

Sasaki, published by Shujun-sha, Tokyo (1995), ISBN 4-87962-144-7, pp. 141-146.

The present detection method or the present amplification method as described above can also be used for identifying a raffinose synthase gene or a gene fragment having a partial nucleotide sequence thereof and then isolating and purifying the identified gene or gene fragment thereof to obtain the present gene (hereinafter referred to simply as the present gene acquisition method).

The present gene or the present gene fragment can be obtained, for example, by detecting a probe consisting of the present gene fragment hybridized to DNA in the organism-derived gene library by the present detection method as described above to identify DNA having a nucleotide sequence homologous with the probe used; purifying a clone carrying the DNA; and isolating and purifying plasmid or phage DNA from the clone. When the DNA thus obtained is a gene fragment having a partial nucleotide sequence of the raffinose synthase gene, further screening of the gene library by the present gene detection method using the DNA as a probe gives the present gene in full length.

The present gene or the present gene fragment can be identified, for example, by effecting polymerase chain reaction using a primer having the nucleotide sequence of the present gene fragment to amplify a DNA fragment from the organism-derived DNA by the present amplification method as described above; and then constructing a restriction endonuclease map or determining a nucleotide sequence for the amplified DNA fragment. Based on the nucleotide sequence of the gene fragment obtained, an antisense primer is synthesized for the analysis of 5'-terminal sequences, and a sense primer is synthesized for the analysis of 3'-terminal sequences. The nucleotide sequence of the present gene in full length can be determined by the RACE method using these primers and a commercially available kit, e.g., Marathon Kit of Clontech. The present gene in full length can be obtained by synthesizing new primers based on both terminal sequences in the nucleotide sequence thus determined and effecting polymerase chain reaction again.

The present gene acquisition method as described above makes it possible to

obtain raffinose synthase genes as the present gene from various organisms. For example, a gene coding for a raffinose synthase having an amino acid sequence that has about 50% or higher homology, in the region corresponding to the length of 400 or more amino acids, with the amino acid sequence of SEQ ID NO:1, and capable of producing raffinose by combining a D-galactosyl group through an $\alpha(1\rightarrow6)$ bond with a hydroxyl group attached to the carbon atom at position 6 of a D-glucose residue in a sucrose molecule. More specifically, for example, a raffinose synthase gene having the nucleotide sequence of SEQ ID NO:4 can be obtained by amplifying and identifying a DNA fragment containing a gene fragment having a partial nucleotide sequence of the raffinose synthase gene by the present amplification method using primers designed from the amino acid sequence of SEQ ID NO:1 and soybean cDNA as a template; isolating and purifying the identified DNA fragment, followed by the above procedures to obtain a full-length gene containing the DNA fragment.

A chimera gene comprising the present gene and a promoter linked thereto (hereinafter referred to simply as the present chimera gene) can be constructed.

The promoter to be used is not particularly limited, so long as it is functionable in a host organism to be transformed. The promoter may include, for example, synthetic promoters functionable in *Escherichia coli*, such as *E. coli* lactose operon promoter, *E. coli* tryptophan operon promoter and tac promoter; yeast alcohol dehydrogenase gene (ADH) promoter, adenovirus major late (Ad.ML) promoter, SV40 early promoter, and baculovirus promoter.

When the host organism is a plant or a cell thereof, the promoter may include, for example, T-DNA derived constitutive promoters such as nopaline synthase gene (NOS) promoter and octopine synthase gene (OCS) promoter; plant virus-derived promoters such as cauliflower mosaic virus (CaMV) derived 19S and 35S promoter; derived promoters such as phenylalanine ammonia-lyase (PAL) gene promoter, chalcone synthase (CHS) gene promoter and pathogenesis-related protein (PR) gene promoter. Furthermore, vector pSUM-GY1 (see JP-A 06-189777/1994) can also be used, which

has a promoter giving specific expression in a specified plant tissue, e.g., a promoter of soybean-derived seed storage protein glycinin gene. The use of a chimera gene constructed so as to have such a promoter makes it possible to increase or decrease the content of raffinose family oligosaccharides in a specified tissue of a plant.

The present chimera gene is then introduced into a host organism according to an ordinary gene engineering method to give a transformant. If necessary, the present chimera gene may be used in the form of a plasmid, depending upon the transformation method for introducing the gene into the host organism. Furthermore, the present chimera gene may contain a terminator. In this case, it is generally preferred that the chimera gene is constructed so as to have a terminator downstream the raffinose synthase gene. The terminator to be used is not particularly limited, so long as it is functionable in a host organism to be transformed. For example, when the host organism is a plant or a cell thereof, the terminator may include, for example, T-DNA derived constitutive terminators such as nopaline synthase gene (NOS) terminator; and plant derived terminators such as terminators of allium virus GV1 or GV2.

If necessary, the present gene may be used in the form of a plasmid. For example, when the host organism is a microorganism, the plasmid constructed is introduced into the microorganism by an ordinary means, for example, as described in "Molecular Cloning: A Laboratory Manual 2nd edition" (1989), Cold Spring Harbor laboratory Press or "Current Protocols in Molecular Biology" (1987), John Wiley & Sons, Inc., ISBN 0-471-50338-X. The microorganism thus transformed is selected with a marker such as antibiotic resistance or auxotrophy. When the host organism is a plant, the plasmid constructed is introduced into a plant cell by an ordinary means such as infection with Agrobacterium (see JP-B 2-58917/1990 and JP-A 60-70080/1985), electroporation into protoplasts (see JP-A 60-251887/1985 and JP-B 5-68575/1993) or particle gun method (see JP-A 5-508316/1993 and JP-A 63-258525/1988). The plant cell transformed by the introduction of a plasmid is selected with an antibiotic such as kanamycin or hygromycin. From the plant cell thus transformed, a transformed plant can

be regenerated by an ordinary plant cell cultivation method, for example, as described in "Plant Gene Manipulation Manual (How to Produce Transgenic Plants)" written by Uchimiya, 1990, Kodan-sha Scientific (ISBN 4-06-153513-7), pp. 27-55. Furthermore, the collection of seeds from the transformed plant also makes it possible to prolify the transformed plant. In addition, crossing between the transformed plant obtained and the non-transformed plant makes it possible to produce progenic plants with the character of the transformed plant.

The content of raffinose family oligosaccharides can be changed by introducing the present gene into a host organism or a cell thereof, and modifying the metabolism in the host organism or the cell thereof. As such a method, for example, there can be used a method for metabolic modification to increase the amount of raffinose family oligosaccharides in a host organism or a cell thereof by constructing the present chimera gene comprising the present gene and a promoter linked thereto, in which case the present gene is linked to the promoter in an original direction suitable for transcription, translation, and expression as a protein, and then introducing the present chimera gene into the host organism or the cell thereof; or a method for metabolic modification to decrease the amount of raffinose family oligosaccharides in a host organism or a cell thereof by constructing the present chimera gene comprising the present gene and a promoter linked thereto, in which case the present gene is linked to a promoter in a reverse direction unsuitable for translation and expression as a protein, and then introducing the present chimera gene into the host organism or the cell thereof.

The term "raffinose synthase protein" as used herein refers to a protein encoded in the present gene (hereinafter referred to simply to the present protein). For example, it may include an enzyme protein having the amino acid sequence of SEQ ID NO:1 or SEQ ID NO: 3, or having an amino acid sequence derived by deletion, replacement, modification or addition of one or several amino acids in the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:3; and capable of producing raffinose by combining a D-galactosyl group through an $\alpha(1\rightarrow6)$ bond with a hydroxyl group attached to the

carbon atom at position 6 of a D-glucose residue in a sucrose molecule.

Specific examples of the present protein are an enzyme protein having the amino acid sequence of SEQ ID NO:1 (799 amino acids; molecular weight, 89 kDa) and an enzyme protein having the amino acid of SEQ ID NO:3 (781 amino acids; molecular weight, 87 kDa).

The present protein, although it can be prepared, for example, from leguminous plants such as broad bean (*Vicia faba*), by an ordinary biochemical method such as $(NH_4)_2SO_4$ precipitation, ion exchange column, hydrophobic column, hydroxyapatite column or gel filtration column, can also be prepared from the host organism transformed with the present plasmid, or a cell thereof. More specifically, for example, using GST Gene Fusion Vectors Kit of Pharmacia, the present gene is inserted into an expression vector plasmid attached to the kit. The resulting vector plasmid is introduced into a microorganism such as *E. coli* according to an ordinary gene engineering method. A culture of the transformant obtained is grown on a medium with the addition of IPTG (isopropylthio-β-D-galactoside), so that the present protein can be expressed and derived as a fused protein in the culture. The fused protein expressed and induced can be isolated and purified by an ordinary method such as disruption of bacterial cells, column operation or SDS-PAGE electrophoresis. The digestion of the fused protein with a protease such as thrombin or blood coagulation factor Xa gives the present protein. This may preferably be made, for example, according to the method described in "Current Protocols In Protein Science" (1995), John Wiley & Sons, Inc. ISBN 0-471-11184-8. The activity of the present protein can be measured, for example, by the method described in L. Lehle and W. Tanner, Eur. J. Biochem., 38, 103-110 (1973).

An anti-raffinose synthase antibody capable of binding to a raffinose synthase protein (hereinafter referred to simply as the present antibody) can be produced by an ordinary immunological method using the present protein prepared above, as an antigen. More specifically, the present antibody can be produced, for example, according to the method described in Ed Harlow and David Lane, "Antibodies: A Laboratory Manual"

(1988), Cold Spring Harbor Laboratory Press, ISBN 0-87969-314-2.

The present protein can be detected by treating test proteins with the present antibody and detecting a protein having the present antibody bound specifically thereto. Such a detection method can be carried out according to an immunological technique such as Western blot method or enzyme-linked immunosorbent assay (ELISA), for example, as described in Ed Harlow and David Lane, "Antibodies: A Laboratory Manual" (1988), Cold Spring Harbor Laboratory Press.

The Western blot method is carried out, for example, as follows: Proteins are extracted from a plant, for example, according to the method described in Methods in Enzymology, volume 182, "Guide to Protein Purification," pp. 174-193, ISBN 9-12-182083-1. The composition of an extraction buffer can suitably be changed depending upon the plant tissue used. The proteins extracted are electrophoresed according to an ordinary SDS-PAGE method. The proteins electrophoresed in the gel are transferred to a membrane by Western blotting with an ordinary electrical method. More specifically, for example, the gel is immersed in a transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) for 10 minutes, and then placed onto a PVDF membrane cut into the same size as that of the gel. The gel together with the membrane is set in a commercially available transfer apparatus of the semi-dry type. Blotting is carried out at a constant current of 0.8 to 2 mA/cm^2 for 45 minutes to 1 hour. The proteins transferred to the membrane can be detected immunologically with a kit for Western blot detection using a primary antibody, and a secondary antibody or protein A, which has been labeled with alkaline phosphatase or horseradish peroxidase. At this time, the present protein on the membrane can be detected by the use of the present antibody as a primary antibody.

In the ELISA method, for example, the property of proteins binding to the surface of a 96-well ELISA plate made of a resin is utilized in principle for the immunological detection of an antigen finally bound to the surface of the ELISA plate. The test proteins are added as a solution and bound to an ELISA plate, followed by blocking, for example, by the addition of PBS containing a protein such as 5% bovine serum albumin.

Thereafter, the well is washed with PBS, to which a solution containing the present antibody is added to effect the reaction. After the well is washed, a solution containing a secondary antibody labeled with alkaline phosphatase or horseradish peroxidase is further added to the well, followed by washing. Finally, a substrate solution for detection is added to the well, and the color development of the substrate is detected with an ELISA reader.

In another method, the present antibody is added and bound to an ELISA plate, followed by blocking, for example, by the addition of PBS containing a protein such as 5% bovine serum albumin. The test proteins are then added as a solution, and an antigen contained in the test proteins is bound to the present antibody that has been bound to the plate, followed by washing, and the present antibody is further added to the well. The present antibody used at this time is preferably one prepared from an animal species different from that used for the preparation of the present antibody used first. A solution containing a secondary antibody labeled with alkaline phosphatase or horseradish peroxidase is then added to the well, followed by washing. The secondary antibody used at this time must have the property of binding to the present antibody added later. Finally, a substrate solution for detection is added, and the color development is detected with an ELISA reader.

Examples

The present invention will be further illustrated by the following examples; however, the present invention is not limited to these example in any way whatsoever.

Example 1 (Purification of Galactinol)

About 250 ml of sugar beet blackstrap molasses was five-fold diluted with methanol. The dilution was centrifuged at 21,400 x g for 15 minutes at room temperature to remove insoluble matter. The supernatant obtained was transferred into a 2-liter Erlenmeyer flask, to which isopropanol at a half volume was added portionwise with stirring. The flask was left at room temperature for a while until the resulting precipitate adhered to the wall of the flask. The supernatant was then discarded by decantation. To

the precipitate was added 500 ml of ethanol, and the mixture was washed by stirring with a rotary shaker. The washing was further repeated several times. The washed precipitate was scraped off from the wall of the flask, followed by air drying on a filter paper. The air-dried precipitate (dry powder) was dissolved in purified water to about 40% (w/v). To this solution was added AG501-X8(D) of BioRad, followed by stirring. This operation was repeated until the color of the solution became almost unobserved. The resulting solution was treated with a Sep-Pak QMA column of Millipore, and further pretreated with Sep-Pak CM, Sep-Pak C18 and Sep-Pak Silica columns of Millipore. The resulting solution was loaded at a volume of 5 ml onto a column of Wako-gel LP40C18 (Wako Pure Chemical Industries, 2.6 cm x 85 cm), and eluted with purified water. The sugar content of the eluate was measured with a portable sugar refractometer, and the sugar composition was analyzed by high performance liquid chromatography (HPLC) with a Sugar-pak Na (7.8 mm x 300 mm) column of Millipore. The detection of sugars was carried out with model 410 Differential Refractometer of Waters. The eluate containing galactinol was lyophilized, and the resulting lyophilized powder was dissolved in 5 ml of purified water. The solution was loaded onto a column of TOYOPEARL HW40(S) (Toso, 2.6 cm x 90 cm), and eluted with purified water. The eluate was analyzed in the same manner as described above, so that purified galactinol was obtained.

The galactinol obtained was kept at 25°C for 40 minutes in the reaction mixture that came to contain 80 mM phosphate buffer (pH 6.5), 2 mg/ml galactinol, and 8.3 U α-galactosidase (Boehringer Mannheim, *E. coli* overproducer 662038). The reaction mixture was extracted with chloroform, and the water layer was analyzed by HPLC. The resulting galactinol was confirmed to be hydrolyzed into galactose and myo-inositol.

Example 2 (Measurement of Raffinose Synthase Activity)

The raffinose synthase activity was measured under the following conditions according to the description of L. Lehle and W. Tanner, Eur. J. Biochem., 38, 103-110 (1973).

First, 2 μl of a sample to be used in the measurement of activity was added to 18 μl of the reaction mixture that came to contain 100 mM Tris-HCl (pH 7.4), 5 mM DTT (dithiothreitol), 0.01% BSA, 200 μM sucrose, 5 mM galactinol, 740 KBq/ml (31.7 μM) [^{14}C] sucrose, and the reaction mixture was kept at 37°C for 3 to 20 hours. After the reaction, 30 μl of ethanol was added to the reaction mixture, followed by stirring and centrifugation at 15,000 rpm for 5 minutes. The supernatant was spotted at a volume of 5 μl on an HPTLC plate of cellulose for thin layer chromatography (Merck, 10 cm x 20 cm), and developed with n-butanol : pyridine : water : acetic acid = 60 : 40 : 30 : 3. The developed plate was dried and then quantitatively analyzed with an imaging analyzer (Fuji Photographic Film, FUJIX Bio Imaging Analyzer BAS-2000II) for the determination of [^{14}C] raffinose produced.

Example 3 (Purification of Raffinose Synthase)

The purification of raffinose synthase from broad bean was carried out as follows: For each purified protein solution, proteins present in the protein solution were analyzed by SDS-PAGE (Daiichi Kagaku Yakuhin), and the enzyme activity thereof was measured according to the method described in Example 2.

First, 300 g of immature seeds of broad bean (Nintoku Issun) stored at –80°C was thawed and then peeled. The peeled seeds were put in 600 ml of 100 mM Tris-HCl (pH 7.4), 5 mM DTT (dithiothreitol), 1 mM EDTA, 1 mM PMSF (phenylmethylsulfonyl fluoride) and 1 mM benzamide, and ground on ice with a mortar. The ground material was centrifuged at 21,400 x g for 50 minutes at 4°C. To the resulting supernatant was added 10% polyethylene imine (pH 8.0) at a 1/20 volume. The mixture was stirred at 4°C for 15 minutes, and centrifuged at 15,700 x g for 20 minutes at 4°C. To the resulting supernatant was added 196 g/l of $(NH_4)_2SO_4$ with stirring. The mixture was stirred in ice for 30 minutes, and centrifuged at 15,700 x g for 20 minutes at 4°C. To the resulting supernatant was further added 142 g/l of $(NH_4)_2SO_4$ with stirring. After the stirring in ice for 30 minutes, the mixture was centrifuged at 15,700 x g for 20 minutes at 4°C. The resulting precipitate was dissolved in 50 ml of 100 mM Tris-HCl (pH 7.4) and 5 mM

DTT (dithiothreitol), and the solution was dialyzed against 20 mM Tris-HCl (pH 7.4), 1 mM DTT (dithiothreitol) and 1 mM EDTA at 4°C overnight. After the dialysis, the suspension was centrifuged at 70,000 x g for 60 minutes at 4°C. To the resulting supernatant was added 1 mM benzamidine · HCl, 5 mM ε-amino-n-caproic acid, 1 μg/ml antipain, 1 μg/ml leupeptin and 10 mM EGTA, and 2 M KCl was further added portionwise at a 1/40 volume. The mixture was loaded onto a column of DEAE-Sephacel (Pharmacia, 2.5 cm x 21.5 cm) equilibrated with 0.05 M KCl, 20 mM Tris-HCl (pH 7.4), 1 mM DTT (dithiothreitol) and 1 mM EDTA, and the adsorbed proteins were eluted with a gradient of 0.05 to 0.5 M KCl. The purification steps up to this stage were repeated three times, and fractions having raffinose synthase activity were combined and then purified as follows:

To the eluted fraction having raffinose synthase activity was added portion-wise saturated $(NH_4)_2SO_4$ at a 1/4 volume. The solution was loaded onto a column of Phenyl-Sepharose (Pharmacia, 2.5 cm x 10.2 cm) equilibrated with 20% saturated $(NH_4)_2SO_4$, 20 mM Tris-HCl (pH 7.4), 1 mM DTT (dithiothreitol) and 1 mM EDTA, and the adsorbed proteins were eluted with a gradient of 20% to 0% $(NH_4)_2SO_4$. The resulting active fraction was diluted by the addition of 0.01 M potassium phosphate buffer (pH 7.5) at a 2-fold volume. The diluted solution was loaded onto a column of Econo-Pac 10DG (BioRad, 5 ml) previously equilibrated with 0.01 M potassium phosphate buffer (pH 7.5) and 2 mM DTT (dithiothreitol), and the adsorbed proteins were eluted with a gradient of 0.01 to 0.5 M potassium phosphate buffer (pH 7.5) and 2 mM DTT (dithiothreitol). The active fraction obtained at this stage was found to have been purified up to 6500-fold or higher specific activity. Part of the resulting purified protein solution having raffinose synthase activity was loaded onto a column of Superdex 200 (Pharmacia, 1.6 cm x 60 cm) equilibrated with 0.2 M KCl, 20 mM Tris-HCl (pH 7.4), 1 mM DTT (dithiothreitol) and 1 mM EDTA. The purified proteins thus separated were subjected to SDS-PAGE, and the raffinose synthase activity was measured. A protein band having raffinose synthase activity was identified as having a molecular weight of about 90 kDa on

the SDS-PAGE.

Example 4 (Analysis of Partial Amino Acid Sequence of Raffinose Synthase)

To about 1 ml of the purified protein solution, which had been purified with a column of Econo-Pac 10DG (BioRad, 5 ml) in Example 3, was added 100% TCA at a 1/9 volume, and the mixture was left on ice for 30 minutes. After centrifugation at 10,000 x g for 15 minutes, the resulting precipitate was suspended in 500 μl of cold acetone (–20°C), followed by further centrifugation. This acetone washing was repeated, and the collected precipitate was dried and then dissolved in 200 μl of SDS-sample buffer, followed by SDS-PAGE. CBB staining was effected for the electrophoresed gel, from which the band of a raffinose synthase protein was cut out.

To the gel thus taken was added 1 ml of 50% acetonitrile and 0.2 M ammonium carbonate (pH 8.9), and washing was continued with stirring at room temperature for 20 minutes. The gel was washed once again in the same manner, and dried under reduced pressure to an extent giving a volume reduction. To this gel was 1 ml of 0.02% Tween-20 and 0.2 M ammonium carbonate (pH 8.9), and the mixture was stirred at room temperature for 15 minutes. After removal of the solution, 400 μl of 8 M urea and 0.4 M NH_4HCO_3 was added, to which 40 μl of 45 mM DTT (dithiothreitol) was further added, and the mixture was left at 50°C for 20 minutes. After complete return to room temperature, 4 μl of 1 M iodoacetic acid was added, and the mixture was stirred in the dark at room temperature for 20 minutes. After removal of the solution, 1 ml of purified water was added, and the mixture was stirred at room temperature for 5 minutes, followed by washing. After further two washings, 1 ml of 50% acetonitrile and 0.2 M ammonium carbonate (pH 8.9) was added, and the mixture was stirred at room temperature for 15 minutes. The same treatment was repeated once again, after which the solution was removed, and the gel was dried under reduced pressure to an extent giving a volume reduction.

To this gel was added a solution of Achromobacter Protease I (Takara, Residue-specific Protease Kit) at a volume of 100 μl. Further added was 0.02%

Tween-20 and 0.2 M ammonium carbonate (pH 8.9) to an extent that the gel was not exposed from the surface of the solution, and the mixture was left at 37°C for 42 hours. Further added was 500 μl of 0.09% TFA and 70% acetonitrile, and the mixture was stirred at room temperature for 30 minutes. The resulting mixture as contained in a sample tube was floated in an ultrasonic bath, followed by ultrasonic treatment (BRANSON, 60 W output power) for 5 minutes. The tube and contents thus treated were centrifuged, and the resulting extract was collected in another silicone-coated sample tube. On the other hand, 500 μl of 0.09% TFA and 70% acetonitrile was added again to the precipitate, followed by repeated extraction in the same manner as described above. The resulting extracts were combined and then concentrated under reduced pressure to an extent giving a solution remained at a volume of 200 to 300 μl. To the concentrate was added 25 μl of 8 M urea and 0.4 M NH_4HCO_3, and the mixture was concentrated to an extent giving a solution remained at a volume of 100 μl or lower. The concentrate was brought to about 100 μl with purified water, and the mixture was filtered through a filter of Ultrafree C3 GV (Millipore). The filtrate obtained was then subjected to elution through a column of Aquapore BU-300 C-4 (2.1 mm x 300 mm) by a gradient of 0.1% TFA/2.1% to 68.6% acetonitrile. Absorbance at 215 nm was monitored to collect a fraction at a peak thereof. The sample collected was evaporated under reduced pressure to complete dryness, and then analyzed with a Protein Sequencer 473A of ABI to determine a partial amino acid sequence of a raffinose synthase.

Example 5 (Preparation of cDNA)

About 2 g of immature seeds of broad bean (Nintoku Issun) was frozen in liquid nitrogen and then ground with a mortar, to which 20 ml of Isogen (Nippon Gene) was added, and the mixture was further thoroughly ground. The ground material was transferred into a centrifugation tube, to which 4 ml of chloroform was added, and the mixture was stirred with a vortex mixer and then centrifuged at 6,500 x g for 10 minutes at 4°C. The water layer was collected, to which 10 ml of isopropanol was added, and the mixture was stirred and then centrifuged at 6,500 x g for 10 minutes at 4°C. The resulting

precipitate was washed with 10 ml of 70% ethanol and then dissolved in 1 ml of elution buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.1% SDS). The solution was left at 60°C for 10 minutes and then centrifuged at 10,000 x g for 1 minute to remove insoluble matter. To the resulting supernatant was added an equivalent volume of Oligotex-dT30 (Takara), and the mixture was stirred and then left at 65°C for 5 minutes. The mixture was placed on ice and then left for 3 minutes, to which 200 μl of 5 M NaCl was added, and the mixture was left at 37°C for 10 minutes. The mixture was then centrifuged at 10,000 x g at 4°C for 3 minutes. The precipitate was collected and then suspended in 1 ml of TE buffer, and the suspension was left at 65°C for 5 minutes, which was placed on ice and then left for 3 minutes, followed by centrifugation at 10,000 x g for 3 minutes at 4°C to remove the precipitate.

To the resulting supernatant were added 100 μl of 3 M sodium acetate and 2 ml of ethanol, and RNA was ethanol precipitated and collected. The collected RNA was washed twice with 70% ethanol and then dissolved in 20 μl of sterilized water, which was used for the subsequent cDNA synthesis. The amount of RNA obtained was determined by the measurement of absorbance at 260 nm.

For the cDNA synthesis, First Strand Synthesis Kit for RT-PCR (Amercham) and cDNA Synthesis Kit (Takara) were used, and all operations were made according to the protocol.

Example 6 (Nucleotide sequence Analysis of Raffinose Synthase Gene from cDNA)

Based on the amino acid sequence obtained in Example 4, mixed synthetic DNA primers having the nucleotide sequences shown in list 5 below were synthesized. The PCR method was carried out with Gene Amp PCR Systems 2400 and DNA Thermal Cycler Model 480 of Perkin-Elmer using Advantage KlenTaq cDNA Kit of Clontech. The polymerase chain reaction was effected with the above primers at 94°C for 1 minute, at 50°C for 3 minutes, and at 72°C for 3 minutes, and this reaction was repeated forty times. As a result, the combinations of primers 8.2 and 13.3RV, primers 13.4 and

10.3RV, and primers 7.4 and 10.3RV, having the nucleotide sequences shown in list 5 below, gave an amplification of 1.2 kb, 0.5 kb, and 1.2 kb bands, respectively. These amplified DNA fragments were cloned with a TA cloning kit (Invitrogen), followed by sequence reaction using ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit of Perkin-Elmer and nucleotide sequence analysis with a 373S DNA sequencer of ABI. As a result, these DNA fragments were found to have a nucleotide sequence extending from base 813 to base 1915, base 1936 to base 2413, and base 1226 to base 2413, respectively, in the nucleotide sequence of SEQ ID NO:2. Based on these nucleotide sequences, synthetic DNA primers having nucleotide sequences shown in list 6 below were prepared, and the nucleotide sequences in both terminal regions of cDNA were analyzed with Marathon cDNA Amplification Kit of Clontech. As a result, the nucleotide sequence of SEQ ID NO:2 was finally determined.

(List 5)

#8.2 26mer (SEQ ID NO:48)

AA(AG) AC(ATGC) GC(ATGC) CC(ATGC) AG(TC) AT(TCA) AT(TCA) GAC AA

#13.4 20mer (SEQ ID NO:49)

AA(AG) AT(TCA) TGG AA(TC) CT(ATGC) AAC AA

#7.4 24mer (SEQ ID NO:50)

AA(AG) GC(ATGC) AG(AG) GT(ATGC) GT(ATGC) GT(ATGC) CC(ATGC) AAG

#13.3RV 21mer (SEQ ID NO:51)

(TC)TT (AG)TT (ATGC)AG (AG)TT CCA (AGT)AT TTT

#10.3RV 21mer (SEQ ID NO:52)

(TC)TT (AG)TC (TC)TC (AG)TA (ATGC)AG (AG)AA TTT

(List 6)

RS-2RV 30mer (SEQ ID NO:53)

GGCTGAGGTTCGGTTCATTCCTGAATCATC

RS-7 30mer (SEQ ID NO:54)

CCAAATGGTACATATTGGCTCCAAGGTTGT

RS-8 30mer (SEQ ID NO:55)

AAGAGTGTATCTGAATTTTCACGCGCGGTG

RS-9 30mer (SEQ ID NO: 56)

TGGTGCAATGGGAAAACTCCAATGAGCACC

RS-10 30mer (SEQ ID NO: 57)

ATGAAGTGTTCTGATAGATTGAAAGTTTCG

RS-11 30mer (SEQ ID NO: 58)

CAGTCTCTGGAGTTTGATGATAATGCAAGT

Example 7 (Cloning of Raffinose Synthase Gene from Broad Bean cDNA)

The primers designed from the amino acid sequence of SEQ ID NO:1, i.e., primers having nucleotide sequences shown in list 7 below, were synthesized. Using these primers and cDNA obtained in Example 5 as a template, a DNA fragment of the open reading frame region was amplified by PCR under the conditions described in Example 6. The amplified DNA fragment was digested with the restriction endonucleases *Bam* HI and *Xba* I whose recognition sequences were contained in the primers used. Using Ligation Kit (Takara), the DNA fragment thus digested was cloned in the plasmid pBluescriptII KS- (Stratagene) previously digested with *Bam* HI and *Xba* I. The nucleotide sequence of the cloned DNA fragment was confirmed with ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit of Perkin-Elmer. In the clone thus obtained, it was found that the base at position 1591 in the nucleotide sequence of SEQ ID NO:2 had been changed from thymine (T) to cytosine (C). This was, however, a nonsense mutation without a change of the amino acid; therefore, this clone was designated pBluescriptKS-RS, and used in the subsequent experiment.

(List 7)

RS-N 41mer (SEQ ID NO: 59)

CGCGGATCCACCATGGCACCACCAAGCATAACCAAAACTGC

RS-C 37mer (SEQ ID NO: 60)

TGCTCTAGATTATCAAAATAAAAACTGGACCAAAGAC

Example 8 (Expression of Broad Bean Raffinose Synthase Gene in *E. coli*)

The plasmid pBluescriptKS-RS having the broad bean raffinose synthase

gene obtained in Example 7 was digested with *Bam* HI and *Not* I, and cloned in the plasmid pGEX-4T3 (Pharmacia) digested with *Bam* HI and *Not* I to give the plasmid pGEX-RS as shown in Figure 1.

The plasmid pBluescriptKS-RS was digested with *Nco* I and *Xba* I, and cloned in the plasmid pTrc99A (Pharmacia) digested with *Nco* I and *Xba* I to give the plasmid pTrc-RS as shown in Figure 1.

These plasmids were introduced into *E. coli* strain HB101, and the resulting transformants were used for the confirmation of raffinose synthase expression. Overnight cultures of the transformants were inoculated at a volume of 1 ml each into 100 ml of LB medium and incubated at 37°C for about 3 hours, followed by the addition of IPTG (isopropylthio-β-D-galactoside) to a final concentration of 1 mM and further incubation for 5 hours. The cultures were centrifuged at 21,400 x g for 10 minutes, and the bacterial cells were collected. The collected bacterial cells were stored at –80°C. To the frozen bacterial cells was added a 10-fold volume of 100 mM Tris-HCl (pH 7.4), 1 mM EDTA, 5 mM DTT (dithiothreitol), 1 mM PMSF (phenylmethylsulfonyl fluoride) and 1 mM benzamide, and the bacterial cells were thawed and suspended. These suspensions were treated with an ultrasonic disrupter (Branson) to effect the disruption of the bacterial cells. The disrupted cell mixtures obtained were centrifuged at 16,000 x g for 10 minutes, and soluble protein solutions were collected.

The protein solutions thus obtained were used at a volume of 4 μl each for the measurement of raffinose synthase activity according to the method described above. The reaction was effected at 37°C for 64 hours. As a control, *E. coli* strain HB101 that had been transformed with one of the vectors, pGEX-4T3, was used. The results are shown in Table 1. The synthesis of raffinose was detected in the samples from the transformants HB101 (pGEX-RS) and HB101 (pTrc-RS).

TABLE 1

Transformant	Amount of raffinose produced (pmol)
HB101 (pGEX4T-3)	0.56
HB101 (pGEX-RS)	10.50
HB101 (pTrc-RS)	11.10

Example 9 (Cloning of Raffinose Synthase Gene from Soybean cDNA)

In the same manner as described in Example 5, cDNA was obtained from immature seeds of soybean (*Glycine max*) Williams 82. Using this cDNA as a template and primers designed from the amino acid sequence of SEQ ID NO:1, i.e., primers having nucleotide sequences shown in list 8 below, a DNA fragment was amplified by PCR under the conditions described in Example 6. The DNA fragment thus amplified by PCR was cloned with a TA cloning kit (Invitrogen), followed by sequence reaction using ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit of Perkin-Elmer and nucleotide sequence analysis with a 373S DNA sequencer of ABI. Based on this sequence, primers having nucleotide sequences shown in list 9 below were synthesized. The synthesis of cDNA was carried out with Marathon Kit of Clontech using mRNA obtained in the same manner as described in Example 5 from leaves of soybean Williams 82. The cDNA obtained was ligated to an adaptor contained in this kit with ligase. This operation was made according to the protocol attached. Using the adaptor-ligated cDNA thus prepared, polymerase chain reaction was effected with the primers shown in list 9 below. The nucleotide sequences in both terminal regions of the gene were analyzed according to the protocol attached to the Marathon Kit of Clontech. As a result, the nucleotide sequence of SEQ ID NO:4 was determined.

(List 8)

1-F primer 35mer

CGATTIAAIGTITGGTGGACIACICAITGGGTIGG (SEQ ID NO: 61)

2-RV primer 45mer (SEQ ID NO: 62)

GGCCTAIAAIGCITCCCAIGTICACCAICCIAAITTITCIATIAT

5-F primer 41mer (SEQ ID NO: 63)

CGATGGATGGGIAAITTIATICAICCIGAITGGGAIATGTT

6-RV primer 32mer (SEQ ID NO: 64)

GGCCACATITTIACIA(AG)ICCIATIGGIGCIAA

(List 9)

SN-1 30mer (SEQ ID NO: 65)

CACGAACTGGGGCACGAGACACAGATGATG

SC-3RV 30mer (SEQ ID NO: 66)

AAGCAAGTCACGGAGTGTGAATAGTCAGAG

SC-5 30mer (SEQ ID NO: 67)

ACACGAGACTGTTTGTTTGAAGACCCCTTG

SC-6 25mer (SEQ ID NO: 68)

TGGAATCTCAACAAATATACAGGTG

SN-3RV 30mer (SEQ ID NO: 69)

GGGTCATGGCCAACGTGGACGTATAAGCAC

SN-4RV 30mer (SEQ ID NO: 70)

GATGATCACTGGCGCGGTTTTCTCCTCGAG

Example 10 (Acquisition of Raffinose Synthase Gene from Japanese Artichoke cDNA)

In the same manner as described in Example 5, cDNA was obtained from leaves of Japanese artichoke (*Stachys sieboldii*). Using this cDNA as a template and primers designed from the amino acid sequence of SEQ ID NO:1, i.e., primers having nucleotide sequences shown in list 10 below, a DNA fragment was amplified by PCR under the conditions described in Example 6. The DNA fragment thus amplified by PCR was cloned with a TA cloning kit (Invitrogen), followed by sequence reaction using ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit of Perkin-Elmer and nucleotide sequence analysis with a 373S DNA sequencer of ABI. As a result, the nucleotide sequence of SEQ ID:6 was determined.

Based on the nucleotide sequence thus obtained, synthesized DNA primers

are prepared, and in the same manner as described in Example 9, the nucleotide sequences in both terminal regions of the gene are analyzed with Marathon Kit of Clontech.

(List 10)

1-F primer 35mer (SEQ ID NO: 71)

CGATTIAAIGTITGGTGGACIACICAITGGGTIGG

4-RV primer 37mer (SEQ ID NO: 72)

GGCCAGCIATIACICCITTICCITTIAAITGITTITT

2-F primer 44mer (SEQ ID NO: 73)

CGAATIATIGAIAAITTIGGITGGTGIACITGGGAIGCITTITA

6-RV primer 32mer (SEQ ID NO: 74)

GGCCACATITTIACIA(AG)ICCIATIGGIGCIAA

Example 11 (Acquisition of Raffinose Synthase Gene from Corn cDNA)

In the same manner as described in Example 5, cDNA was obtained from leaves of corn (*Zea mays* L.) Pioneer 3358. Using this cDNA as a template and primers designed from the amino acid sequence of SEQ ID NO:1, i.e., primers having nucleotide sequences shown in list 11 below, a DNA fragment was amplified by PCR under the conditions described in Example 6. The DNA fragment thus amplified by PCR was cloned with a TA cloning kit (Invitrogen), followed by sequence reaction using ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit of Perkin-Elmer and nucleotide sequence analysis with a 373S DNA sequencer of ABI. Based on this sequence, primers having nucleotide sequences shown in list 12 below were synthesized. In the same manner as described in Example 5, mRNA obtained from leaves of corn (*Zea mays* L.) Pioneer 3358 was linked to an adaptor contained in the Marathon Kit of Clontech with ligase. This operation was made according to the protocol attached. Using the adaptor-ligated cDNA thus prepared, polymerase chain reaction was effected in the same manner as described above with the primers shown in list 12 below. As a result, the nucleotide sequence of SEQ ID NO:8 was determined.

Based on the nucleotide sequence thus obtained, synthesized DNA primers are prepared, and in the same manner as described in Example 9, the nucleotide sequence

in the 5'-terminal region of the gene is analyzed with Marathon Kit of Clontech.

(List 11)

5-F primer 41mer (SEQ ID NO: 75)

CGATGGATGGGIAAITTIATICAICCIGAITGGGAIATGTT

6-RV primer 32mer (SEQ ID NO: 76)

GGCCACATITTIACIA(AG)ICCIATIGGIGCIAA

(List 12)

M-10 primer 25mer (SEQ ID NO: 77)

GACGTCGAGTGGAAGAGCGGCAAGG

M-11 primer 25mer (SEQ ID NO: 78)

CACCTACGAGCTCTTCGTCGTTGCC

Example 12 (Construction of Expression Vectors in Plant for Chimera Gene, 35S-Broad Bean Raffinose Synthase Gene)

The plasmid pBluescriptKS-RS having the broad bean raffinose synthase gene obtained in Example 7 was digested with the restriction endonucleases *Bam* HI and *Sac* I. Using Ligation Kit (Takara), the DNA fragment thus digested was cloned in the binary vector pBI121 (Clontech) previously digested with *Bam* HI and *Sac* I. The vector thus obtained was designated pBI121-RS.

For an antisense experiment, plasmid pBI121 (Clontech) previously digested with *Bam* HI and *Sac* I was ligated to linkers shown in list 13 below to give pBI121(–). This pBI121(–) was used to prepare pBI121(–)-RS in the same manner as described for the preparation of pBI121-RS above.

A similar vector was prepared with pBI221. The plasmid pBluescriptKS-RS obtained in Example 7 was digested with the restriction endonucleases *Bam* HI and *Sac* I. Using Ligation Kit (Takara), the DNA fragment thus digested was cloned in the vector pBI221 (Clontech) previously digested with *Bam* HI and *Sac* I. The vector thus obtained was designated pBI221-RS.

For an antisense experiment, plasmid pBI221 (Clontech) previously digested

with *Bam* HI and *Sac* I was ligated to linkers shown in list 13 below to give pBI221(–). This pBI221(–) was used to prepare pBI221(–)-RS in the same manner as described for the preparation of pBI221-RS above.

The construction of these expression vectors is shown in Figures 2 and 3.

(List 13)

BamSac-(+) linker 25mer (SEQ ID NO: 79)

GATCGAGCTCGTGTCGGATCCAGCT

BamSac-(–) linker 17mer (SEQ ID NO: 80)

GGATCCGACACGAGCTC

Example 13 (Transformation of Mustard with Broad Bean Raffinose Synthase Gene)

The vectors pBI121-RS and pBI121(–)-RS prepared in Example 12 were used for the transformation of mustard (*Brassica juncia*) by the Agrobacterium infection method.

Agrobacterium tumefaciens (strain C58C1, rifampicin resistant) previously made into a competent state by calcium chloride treatment was transformed independently with two plasmids pBI121-RS and pBI121(–)-RS prepared in Example 12. Selection for transformants was carried out on LB medium containing 50 μg/ml rifampicin and 25 μg/ml kanamycin by utilizing the character of kanamycin resistance conferred by the kanamycin resistance gene (neomycin phosphotransferase, NPTII) of the introduced plasmids.

The transformant Agrobacterium obtained (*Agrobacterium tumefaciens* strain C58, rifampicin resistant) was cultivated on LB medium containing 50 μg/ml rifampicin and 25 μg/ml kanamycin at 28°C for a whole day and night, and the culture was used for the transformation of mustard by the method described below.

The seeds of mustard were aseptically sowed on 1/2 MS medium, 2% sucrose, 0.7% agar. After one week, cotyledons and petioles of sprouting plants were cut out with a scalpel, and transferred to MS medium, 3% sucrose, 0.7% agar, 4.5 μM BA,

0.05 μM 2.4-D, 3.3 μM $AgNO_3$, followed by precultivation for 1 day. The precultivated cotyledons and petioles were transferred in a 1000-fold dilution of the Agrobacterium culture to cause infection for 5 minutes. The infected cotyledons and petioles were transferred again to the same medium as used in the precultivation, and cultivated for 3 to 4 days. The cultivated cotyledons and petioles were transferred to MS medium, 3% sucrose, 4.5 μM BA, 0.05 μM 2.4-D, 3.3 μM $AgNO_3$, 500 mg/l cefotaxim, and sterilized with shaking for 1 day. The sterilized cotyledons and petioles were transferred to MS medium, 3% sucrose, 0.7% agar, 4.5 μM BA, 0.05 μM 2.4-D, 3.3 μM $AgNO_3$, 100 mg/l cefotaxim, 20 mg/l kanamycin, and cultivated for 3 to 4 weeks. The cotyledons and petioles were transferred to MS medium, 3% sucrose, 0.7% agar, 4.5 μM BA, 0.05 μM 2.4-D, 100 mg/l cefotaxim, 20 mg/l kanamycin, and cultivated. The cultivation on this medium was continued with subculturing at intervals of 3 to 4 weeks. When the regeneration of shoots began to occur, these shoots were subcultured on MS medium, 3% sucrose, 0.7% agar, 20 mg/l kanamycin, and cultivated for 3 to 4 weeks. The rooting plants were transferred to vermiculite : peat moss = 1 : 1, and conditioned at 21° to 22°C in a cycle of day/night = 12 hours : 12 hours. With the progress of plant body growth, the plants were suitably grown with cultivation soil. From leaves of the regenerated plants, genomic DNA was extracted according to the method described above, and the gene insertion into the plant genome was confirmed by PCR using the primers shown in list 14 below.

(List 14)

35S 30mer

TTCCAGTATGGACGATTCAAGGCTTGCTTC (SEQ ID NO: 81)

NOS 25mer

ATGTATAATTGCGGGACTCTAATCA (SEQ ID NO: 82)

RS-F 30mer

AAGAGTGTATCTGAATTTTCACGCGCGGTG (SEQ ID NO: 83)

RS-RV 33mer

ACCTTCCCATACACCTTTTGGATGAACCTTCAA (SEQ ID NO: 84)

Example 14 (Transformation of Soybean Somatic Embryo with Broad Bean Raffinose Synthase Gene)

Cultured cells of soybean "Fayette" somatic embryos (400 to 500 mg FW) were arranged in one layer within a circle having a diameter of 20 mm on the central part of a 6 cm agar plate. Two plasmids pBI221-RS and pBI221(–)-RS having chimera genes prepared from the broad bean raffinose synthase gene and 35S promoter in Example 12 were introduced into the soybean somatic embryos according to the disclosure of the Japanese Patent Application No. 3-291501/1991. That is, these plasmids were mixed with the β-glucuronidase (GUS)/hygromycin-resistant gene (HPT) coexpression vector pSUM-GH:NotI for selection described in Soshiki Baiyo, 20, 323-327 (1994). These mixed plasmids were used for the gene introduction into the soybean somatic embryos with a particle gun (800 mg/coating gold particles 200 μg/shot; projectile stopper-sample distance, 100 mm). After the introduction, gyratory cultures were grown in the MS modified growth liquid medium (Sigma) containing 25 to 50 μg/ml hygromycin under illumination at 25°C for 16 hours, and transformed somatic embryos were selected.

For the hygromycin-resistant soybean somatic embryos having yellowish green color and growth ability, which were selected after about 3 months, polymerization chain reaction is effected with primers shown in list 14 above to determine whether the broad bean raffinose synthase gene region is amplified or not. This confirms that the broad bean raffinose synthase gene is inserted into the soybean genome.

Furthermore, the somatic embryos obtained are used for the regeneration of plants to give transformant soybean with the broad bean raffinose synthase gene.

Medium Composition

LB and MS media used in the above Examples have the following respective compositions.

(LB medium)

Bacto-tryptone	10 g
Bacto-yeast extract	5 g

NaCl	10 g	/ 1 liter H_2O (pH 7.0)

(MS medium)

KNO_3	2022 mg/l
NH_4NO_3	1650 mg/l
NH_4Cl	2140 mg/l
KH_2PO_4	170 mg/l
$MgSO_4 \cdot 7H_2O$	370 mg/l
$CaCl_2 \cdot 2H_2O$	440 mg/l
$MnSO_4 \cdot 4H_2O$	22.3 mg/l
$ZnSO_4 \cdot 7H_2O$	8.6 mg/l
$CuSO_4 \cdot 5H_2O$	0.025 mg/l
KI	0.83 mg/l
$CoCl_2 \cdot 6H_2O$	0.025 mg/l
H_3BO_3	6.2 mg/l
NaMoO4 $\cdot 2H_2O$	0.25 mg/l
$FeSO_4 \cdot 7H_2O$	27.8 mg/l
Na_2EDTA	37.3 mg/l
Nicotinic acid	0.5 mg/l
Thiamine HCl	1 mg/l
Pyridoxine HCl	0.5 mg/l
Inositol	100 mg/l
Glycine	2 mg/l

Brief Description of the Sequences

1. SEQ ID NO:1:

The sequence of SEQ ID NO:1 shows an amino acid sequence of a raffinose synthase protein encoded in the raffinose synthase gene obtained from broad bean.

2. SEQ ID NO:2:

The sequence of SEQ ID NO:2 shows a cDNA nucleotide sequence of the raffinose synthase gene obtained from broad bean.

3. SEQ ID NO:3:

The sequence of SEQ ID NO:3 shows an amino acid sequence of a raffinose synthase protein encoded in the raffinose synthase gene obtained from soybean.

4. SEQ ID NO:4:

The sequence of SEQ ID NO:4 shows a cDNA nucleotide sequence of the raffinose synthase gene obtained from soybean.

5. SEQ ID NO:5:

The sequence of SEQ ID NO:5 shows an amino acid sequence of a raffinose synthase protein encoded in the raffinose synthase gene obtained from Japanese artichoke.

6. SEQ ID NO:6:

The sequence of SEQ ID NO:6 shows a cDNA nucleotide sequence of the raffinose synthase gene obtained from Japanese artichoke.

7. SEQ ID NO:7:

The sequence of SEQ ID NO:7 shows an amino acid sequence of a raffinose synthase protein encoded in the raffinose synthase gene obtained from corn.

8. SEQ ID NO:8:

The sequence of SEQ ID NO:8 shows a cDNA nucleotide sequence of the raffinose synthase gene obtained from corn.

9. List 1:

The nucleotide sequences shown in list 1 are of the typical primers used in the amplification of a cDNA fragment of a raffinose synthase gene. All of these sequences are based on the nucleotide sequence in the non-coding region of the gene. Primer 1 is a sense primer corresponding to the 5'-terminus of a cDNA fragment of the broad bean-derived raffinose synthase gene. Primers 2 and 3 are antisense primers corresponding to the 3'-terminus of the cDNA fragment of the broad bean-derived raffinose synthase gene. Primer 4 is a sense primer corresponding to the 5'-terminus of a cDNA fragment of the soybean-derived raffinose synthase gene. Primers 5 and 6 are antisense primers corresponding to the 3'-terminus of the cDNA fragment of the soybean-derived raffinose synthase gene. Depending upon the purpose, recognition sequences for suitable restriction endonucleases can be added to the 5'-termini of these nucleotide sequences in an appropriate manner.

10. List 2:

The nucleotide sequences shown in list 2 are of the typical primers used in the amplification of an open reading frame coding for the amino acid sequence of a raffinose synthase protein in the cDNA sequence of a raffinose synthase gene. Primers 1 and 2 are sense primers corresponding to the N-terminus of the broad bean-derived raffinose synthase protein. Primers 3 and 4 are antisense primers corresponding to the C-terminus of the broad bean-derived raffinose synthase protein. Primers 5 and 6 are sense primers corresponding to the N-terminus of the soybean-derived raffinose synthase protein. Primers 7 and 8 are antisense primers corresponding to the C-terminus of the soybean-derived raffinose synthase protein. Depending upon the purpose, recognition sequences for suitable restriction endonucleases can be added to the 5'-termini of these sequences in an appropriate manner.

11. List 3:

The amino acid sequences shown in list 3 are partial amino acid sequences of a raffinose synthase protein.

#1 is equivalent to the partial amino acid sequence extending from amino acid 110 to amino acid 129 in the amino acid sequence of SEQ ID NO:1.

#2 is equivalent to the partial amino acid sequence extending from amino acid 234 to amino acid 247 in the amino acid sequence of SEQ ID NO:1.

#3 is equivalent to the partial amino acid sequence extending from amino acid 265 to amino acid 279 in the amino acid sequence of SEQ ID NO:1.

#4 is equivalent to the partial amino acid sequence extending from amino acid 296 to amino acid 312 in the amino acid sequence of SEQ ID NO:1.

#5 is equivalent to the partial amino acid sequence extending from amino acid 346 to amino acid 361 in the amino acid sequence of SEQ ID NO:1.

#6 is equivalent to the partial amino acid sequence extending from amino acid 383 to amino acid 402 in the amino acid sequence of SEQ ID NO:1.

#7 is equivalent to the partial amino acid sequence extending from amino acid

411 to amino acid 433 in the amino acid sequence of SEQ ID NO:1.

#8 is equivalent to the partial amino acid sequence extending from amino acid 440 to amino acid 453 in the amino acid sequence of SEQ ID NO:1.

#9 is equivalent to the partial amino acid sequence extending from amino acid 457 to amino acid 468 in the amino acid sequence of SEQ ID NO:1.

#10 is equivalent to the partial amino acid sequence extending from amino acid 471 to amino acid 516 in the amino acid sequence of SEQ ID NO:1.

#11 is equivalent to the partial amino acid sequence extending from amino acid 517 to amino acid 559 in the amino acid sequence of SEQ ID NO:1.

#12 is equivalent to the partial amino acid sequence extending from amino acid 574 to amino acid 582 in the amino acid sequence of SEQ ID NO:1.

#13 is equivalent to the partial amino acid sequence extending from amino acid 586 to amino acid 609 in the amino acid sequence of SEQ ID NO:1.

#14 is equivalent to the partial amino acid sequence extending from amino acid 615 to amino acid 627 in the amino acid sequence of SEQ ID NO:1.

#15 is equivalent to the partial amino acid sequence extending from amino acid 716 to amino acid 724 in the amino acid sequence of SEQ ID NO:1.

12. List 4:

The nucleotide sequences shown in list 4 are of the typical primers synthesized on some of the amino acid sequences shown in list 3. The symbol "F" as used after the primer number means that the primer referred to by this symbol has a sense sequence. The symbol "RV" as used after the primer number means that the primer referred to by this symbol has an antisense sequence. Primer 1 corresponds to the partial amino acid sequence extending from amino acid 119 to amino acid 129 in the amino acid sequence of SEQ ID NO:1. Primer 2 corresponds to the partial amino acid sequence extending from amino acid 234 to amino acid 247 in the amino acid sequence of SEQ ID NO:1. Primer 3 corresponds to the partial amino acid sequence extending from amino acid 265 to amino acid 279 in the amino acid sequence of SEQ ID NO:1. Primer 4 corresponds to the partial

amino acid sequence extending from amino acid 458 to amino acid 468 in the amino acid sequence of SEQ ID NO:1. Primer 5 corresponds to the partial amino acid sequence extending from amino acid 522 to amino acid 534 in the amino acid sequence of SEQ ID NO:1. Primer 6 corresponds to the partial amino acid sequence extending from amino acid 716 to amino acid 724 in the amino acid sequence of SEQ ID NO:1.

13. List 5:

The nucleotide sequences shown in list 5 are of the typical primers synthesized on the partial amino acid sequences of the purified broad bean raffinose synthase protein. The bases shown in parentheses mean that a mixture of those bases was used in the synthesis. The symbol "RV" as used after the primer number means that the primer referred to by this symbol has an antisense sequence.

14. List 6:

The nucleotide sequences shown in list 6 are of the typical primers used in the analysis of both terminal regions of a cDNA nucleotide sequence of the broad bean raffinose synthase gene by the RACE method. The symbol "RV" as used after the primer number means that the primer referred to by this symbol has an antisense sequence.

15. List 7:

The nucleotide sequences shown in list 7 are of the typical primers used in the cloning of the broad bean raffinose synthase gene. RS-N corresponds to the N-terminus of the open reading frame and contains two recognition sites for the restriction endonucleases *Bam* HI and *Nco* I on the 5'-terminal side. RS-C is an antisense primer corresponding to the C-terminus of the open reading frame and contains a recognition site for the restriction endonuclease *Xba* I on the 5'-terminal side.

16. List 8:

The nucleotide sequences shown in list 8 are of the typical primers used in the cloning of a soybean raffinose synthase gene fragment. The base represented by the symbol "I" was inosine used in the synthesis. The symbol "RV" as used after the primer number means that the primer referred to by this symbol has an antisense sequence.

17. List 9:

The nucleotide sequences shown in list 9 are of the typical primers used in the analysis of the cDNA nucleotide sequence of a soybean raffinose synthase gene fragment. The symbol "RV" as used after the primer number means that the primer referred to by this symbol has an antisense sequence.

The analysis of nucleotide sequences was carried out by polymerase chain reaction using SN-1 and SC-3RV. SC-5 and SC-6 were used in the analysis of a nucleotide sequence in the 3'-terminal region, and SN-3RV and SN-4RV were used in the analysis of a nucleotide sequence in the 5'-terminal region.

18. List 10:

The nucleotide sequences shown in list 10 are of the typical primers used in the analysis of the cDNA nucleotide sequence of a Japanese artichoke raffinose synthase gene fragment. The base represented by the symbol "I" was inosine used in the synthesis. The symbol "RV" as used after the primer number means that the primer referred to by this symbol has an antisense sequence.

19. List 11:

The nucleotide sequences shown in list 11 are of the typical primers used in the analysis of the cDNA nucleotide sequence of a corn raffinose synthase gene fragment. The base represented by the symbol "I" was inosine used in the synthesis. The symbol "RV" as used after the primer number means that the primer referred to by this symbol has an antisense sequence.

20. List 12:

The nucleotide sequences shown in list 12 are of the typical primers used in the analysis of the cDNA nucleotide sequence of a corn raffinose synthase gene fragment. M-10 and M-11 were used in the analysis of a nucleotide sequence in the 3'-terminal region.

21. List 13:

The nucleotide sequences shown in list 13 are of the typical adopters used in

the construction of vectors for antisense experiments. These synthetic DNA fragments takes a double-stranded form when mixed together because they are complementary strands. This double-stranded DNA fragment has cohesive ends of cleavage sites for the restriction endonucleases *Bam* HI and *Sac* I on both termini, and contains the restriction sites for the restriction endonucleases *Bam* HI and *Sac* I in the double-stranded region.

22. List 14:

The nucleotide sequences shown in list 14 are of the typical primers used in the PCR experiment to confirm the gene introduction into the genome of a recombinant plant. 35S is a primer toward the downstream region at the 35S promoter site, and NOS is a primer toward the upstream region at the NOS terminator site. RS-F is a sense primer of the broad bean raffinose synthase gene, and RS-RV is an antisense primer of the broad bean raffinose synthase gene.